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(54) Polypeptides corresponding to the amino acid sequences of proteins p57 or p9.5 of Borna disease virus, nucleic acid fragments coding therefore and their use for diagnostic and immunization purposes

(57) The present invention concerns polypeptides corresponding to the amino acid sequence of the protein p57 or p9.5, respectively, encoded by the Borna disease virus. Said polypeptide and isolated DNA and RNA fragments can be used in testkits and for vaccination.

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Description

The present invention relates to the diagnosis and vaccination of a viral infection caused by the Borna disease virus.

Borna disease virus (BDV) is a neurotropic virus that causes an immune-mediated syndrome resulting in disturbances in movement and behaviour. Originally the disease was described as a natural infection of horses in a small city, Borna, in Southeast Germany.

Borna disease (BD) is an infectious disease of the central nervous system characterized by profound behavioural abnormalities, inflammatory cell infiltrates and the accumulation of disease-specific antigens in limbic system neurons. Naturally occurring infections with Borna disease virus (BVD), the etiological agent of Borna disease, have been confirmed mainly in horses and sheep. The disease can, however, be experimentally transmitted to a wide range of animal species including rodents and nonhuman primates with variable clinical and pathological manifestations. Recent epidemiological data suggest that Borna disease may be more widespread in a subclinical form. It is possible that Borna disease virus is involved in human disorders of the central nervous system. Therefore, it is important to have a reliable diagnostic test system and an effective vaccination.

Borna disease virus has not been fully characterized yet, however, the genome of cell adapted Borna disease virus (BDV)-strains have been cloned and sequenced by Cubitt et al. [J. Virol. **68**, p. 1382-1396 (1994)] and Briese et al. [Proc. Natl. Acad. Sci., USA, vol. 91, p. 4362-4366 (May 1994)].

BDV contains a nonsegmented negative-sense 8.9 kb RNA-genome with complementary 3' and 5' termini. Subgenomic RNAs have been mapped to the viral genome and some of them found to undergo posttranscriptional modification by RNA splicing. The features known up to now seem to indicate that BDV represents the prototype of a new group of animal viruses within the order Mononegavirales.

BDV is strictly neurotropic and disseminates by intra-axonal transport from the site of infection. The virus replicates in vitro in embryonic brain cells of various animal species. Cocultivation of such brain cells with various permanent cell lines such as MDCK or Vero cells results in a persistent infection. Infectivity is mainly cell associated, the virus is noncytopathic and spreads by cell to cell contact. Intracellular viral antigen can be demonstrated in the cell nucleus and cytoplasm of infected cells. Morphologically the virion appears to be a 60-90 nm enveloped, spherical particle containing an electron dense internal structure.

BDV replication in cells is associated with the presence of at least three virus-specific antigens with a molecular weight of 18 (gp18), 24 (p24) and 38/40 (p38 or p40) kilodalton. An enzyme-linked immunosorbent assay for detecting antibodies to Borna disease virus by specific proteins is described by Briese et al. (Journal of Clinical Microbiology, **33**, p. 348-351 (February 1995)]. The ELISA test described by Briese uses the proteins p38/40, p23 and gp18 which are found in vitro and in vivo in the nucleus and cytoplasm of infected cells. The recombinant proteins used in the ELISA assay of Briese were produced by using a cell-adapted laboratory BDV strain from persistently BDV-infected MDCK cells.

The disadvantage of the known ELISA test is that only a few BDV proteins are used and therefore not all infections of Borna disease virus can be reliably detected.

In the course of the present invention it has been found that polypeptides corresponding to the proteins p57 and p9.5, respectively, allow a better diagnosis of BDV infection and can be advantageously used for the preparation of vaccines.

The present invention relates therefore to polypeptides corresponding to the amino acid sequence of the protein p57 or p9.5 encoded by the Borna disease virus having a sequence of at least 10 consecutive amino acids of the amino acid sequence given in Figure 1 or Figure 2, respectively.

From the prior art it was not clear whether the proteins p9.5 and p57 are in fact existing or whether they are only hypothetical proteins which are not produced in natural infections with BDV. Neither Briese et al. nor Cubitt et al. confirmed the expression of p57 or p9.5 or provided the isolated proteins.

In the course of the present invention it was found that the protein p9.5 is in fact produced and that this protein which is not glycosylated is located in the nucleus of infected MDCK cells. The protein p57 apparently is a glycosylated protein and the major BDV-specific surface protein which occurs not only in the cytoplasm of infected cells but also in their cell membrane probably determining the tropism of BDV by binding to the respective virus-specific cell receptor. The protein p57 probably also functions as a fusion protein which causes the fusion of an infected cell with another not infected cell. Such fusions allow the spread of the virus from cell to cell. Therefore, this protein is from the therapeutic point of view extremely important, since humoral or cell-mediated immune response directed against such a surface protein with fusion activity can be used for the preparation of an effective vaccine. Probably the protein p57 is modified after the translation by a protease like subtilisin or a furin protease which converts the p57 protein to the active form.

There is another advantage of the polypeptides according to the present invention. Since the sequences of the present invention were obtained from a field isolate of Borna disease virus (from horse), no modifications caused by the permanent culture of the laboratory strain occurred. The sequences of the claimed polypeptide p57 and of p9.5 differ therefore from the corresponding sequence described in the prior art.

The protein p9.5 occurs in the nucleus of persistently BDV-infected MDCK cells and is probably associ-

ated with the nucleic acid of the virus. Therefore, this protein can be advantageously used for the preparation of genomic viral RNA by selectively binding the protein p9.5 to a solid phase. This can be achieved by using affinity chromatography with specific antibodies directed against protein 9.5.

In a preferred embodiment of the present invention the polypeptides comprise the main epitope or main epitopes against which antibodies are formed. Therefore, the polypeptides have preferably a length of at least 25 consecutive amino acids and more preferably of at least 50 consecutive amino acids of the amino acid sequence given in Figure 1 or Figure 2, respectively.

On the other hand the polypeptides according to the invention have preferably an upper limit of not more than 80 consecutive amino acids of the amino acid sequence given in Figure 1 or Figure 2, respectively.

The present invention concerns also testkits for the determination of antibodies directed against Borna disease virus in a sample comprising at least one polypeptide according to the present invention and a label for the detection of the complex formed by the polypeptide and the antibodies to be determined.

The testkits are generally based on the detection of a complex formed by the polypeptide comprising at least one epitope and antibodies directed against said epitope. There are various forms of such testkits whereby the ELISA test is one of the most commonly used tests, because such a test can easily be handled by laboratories. In a preferred embodiment the polypeptide is linked to the surface of the wells of microtiter plates. The sample to be tested which is preferably a serum sample of the individual to be tested is brought into the well and removed after a definite period of time. Afterwards the well is washed and antibodies binding specifically to the polypeptide can be visualized by adding another antibody which specifically binds to the antibody remaining in the well. Said second antibody is usually covalently bound to a label which allows the detection of the complex formed within the test well. Such a label can preferably be selected from enzymes catalyzing a colour reaction as for example horseradish peroxidase.

In preferred embodiments of the present invention the testkits comprise the components for performing an ELISA, Western blot, RIA or dot blot test.

The method according to the invention for determining an infection by Borna disease virus comprises

a) contacting a sample to be determined with at least one polypeptide according to the invention whereby the polypeptide binds to antibodies elicited by a former infection of Borna disease virus and

b) determining the binding of said polypeptide to the specific antibodies which are present in the sample to be tested.

In a further aspect the present invention concerns isolated DNA fragments which encode a polypeptide according to the invention whereby the DNA fragment is preferably not longer than 240 base pairs and more preferably not longer than 150 base pairs.

A further aspect of the present invention concerns isolated RNA fragments which encode a polypeptide according to the invention whereby the RNA fragment is not longer than 240 base pairs.

In preferred embodiments of the present invention the DNA and RNA fragments, respectively, have a sequence which corresponds at least partially to the sequences given in Figure 4 and 5, respectively, or are complementary thereto.

The polypeptides according to the present invention can be used for the production of a vaccine.

The use of proteins, peptides and polypeptides for vaccination has been well-known for a long time. The methods of preparing the vaccine are well-known to those skilled in the art.

There is, however, a further technique for vaccination which can be performed with the nucleic acid fragments of the present invention. It has recently been found that plasmid DNA can be taken up by skeletal muscle cells in vivo without any special delivery mechanism and persist long-term in an extra-chromosomal, nonreplicative circular form. Thus foreign genes can be expressed transiently in skeletal muscle. It is also possible to include the DNA or RNA fragments of the present invention in infectious suicide virus particles which can be used directly for immunization. Furthermore it is also possible to inject the isolated DNA and RNA fragments, respectively, into the muscle of the human or animal to be immunized.

Depending on the form how the DNA fragment is introduced into the individual to be immunized the isolated DNA fragment can further comprise the sequences required for regulation of transcription and expression of the DNA fragment. If the nucleic acid is introduced in a vector, the nucleic acid fragment will be linked to suitable viral vectors or recombinant plasmids.

The DNA fragments and RNA fragments according to the present invention can therefore be used for nucleic acid immunization.

The present invention is further illustrated by the enclosed Figures.

Figure 1 shows the amino acid sequence of the protein p57 (Seq.-ID 1).

Figure 2 shows the amino acid sequence of the protein 9.5 (Seq.-ID 2).

Figure 3 reflects the amino acid sequence of a polypeptide corresponding to the C-terminal region of p57 (Seq.-ID 3).

Figure 4 shows the DNA sequence of p57 (Seq.-ID 4).

Figure 5 corresponds to the DNA sequence of p9.5 (Seq.-ID 5).

Figure 6 shows the results obtained by the ELISA test as described in example 4.

Figure 7 shows the results obtained by the ELISA

test as described in example 10.

Example 1

Cloning and expression of the p57 and p47/c BDV-gene

The entire and the C-terminal region of the open reading frame of the p57 BDV-protein [p57/c; bp 2685-bp 3747, Cubitt et al., (1994) J. Virol. **68**, 7669-7675, Briese et al. (1994) p57 bp x - 3747] was amplified from RNA isolated from BDV-infected rats using the following primers:

C-terminal region:
3' Primer (anti-sense) *GTAGAATTC* **TTATTCCTGCCACCGGCCGAGGCGTC** Seq.-ID 6
entire p57 ORF:
5' Primer (sense): *GATGGATCC* **ATGTACTGCAGTTTCGCGGACTGTAG** Seq.-ID 7
5'-Primer:
RNA was isolated from BDV-infected rat brain using the standard acid guanidium isothiocyanate-phenol-chloroform method and 2 µg RNA was used for RT-reaction. The conditions for the RT-reaction and the PCR were described by Richt et al., [Med. Microbiol. Immunol. 182 (1993) S. 293-304].

The amplified product was purified from agarose gels and cloned into the plasmid vector pGEX-2T (Pharmacia, #27-4801-01) after the restriction sites were cleaved using the restriction enzymes BamHI and EcoRI (Promega, Madison, USA). The viral gene was fused to the Glutathion-S-transferase (GST) gene of *Schistosoma japonicum* controlled by the *tac* promotor. The expression plasmid was transformed into competent *E. coli* Sure™-cells. Recombinant plasmids were analyzed using restriction analysis and DNA-sequencing methods. The amino acid sequence of the fragment p57c deduced from the sequenced DNA fragment is shown in Fig. 3.

Example 2

Expression and purification of the p57 and p57/c BDV-proteins in E. coli:

100 ml of pGEX-p57/c containing *E. coli* were grown overnight in LB-medium with 0.1 mg/ml ampicillin (Serva, Heidelberg). This overnight culture was diluted in 1 liter of LB-medium with ampicillin and grown to log phase for 2-4 hours. The expression of the GST-p57/c and GST-p57 fusion proteins were induced with IPTG (0.1 mM; Promega, Heidelberg, Germany) for 4 hours. The bacteria were pelleted by centrifugation (5900 g, 10 min, 4°C) and resuspended in PBS. The cells were lysed by sonication on ice and the cell debris pelleted by centrifugation (9800 rpm, 10 min, 4°C). The sonicated fusion protein supernatants were added to an affinity matrix with Glutathione (Glutathione Sepharose 4 B; Pharmacia, Nr. 27-4570-01). The purification of the GST-p57/c and GST-p57 fusion proteins using Glutathione Sepharose 4B was done according to the protocol of the manufacturer. The eluted fusion proteins were dialyzed against 1 x PBS for 24 hours at 4°C. The expression product was analyzed in SDS-PAGE and Immunoblot assays.

The expression of the virus-specific GST-p57/c and GST-p57 fusion protein by recombinant pGEX-p.57/c or pGEX-p57 clones were analyzed in immunoblotting using *E.coli* lysates treated with and without IPTG. As a control an *E.coli* lysate transformed with the nonrecombinant pGEX-2T plasmid was used. The quality of the eluted fusion protein was then analyzed in Western blot analyses using BDV-specific rat and rabbit antisera. The purified GST-p57/c as well as GST-p57 were easily detected by virus-specific antisera from rat and rabbit as a distinct band with a MW of ca. 65 or 80 kilodalton, where 26 kd of the fusion protein represent the GST protein and ca. 40 kd or 57 kd represent the C-terminal part of the p57 BDV-protein or the entire p57 BDV-protein.

Example 3

Preparation of antisera and monoclonal antibodies

Polyvalent monospecific antiserum against the GST-p57/c fusion protein was obtained from a rabbit immunized subcutaneously with 1 mg GST-p57/c fusion protein in complete Freund's adjuvant (CFA). After 4 and 8 weeks the rabbit received a booster immunization with the same amount of antigen and was bled 1 week after the last immunization procedure. The serum was tested for its reactivity in indirect immunofluorescence assays on BDV-infected and uninfected MDCK cells as well as in Western blot analyses with the fusion protein.

Monoclonal antibodies were prepared using published procedures (Köhler & Milstein, 1975). Spleen cells were obtained from a Balb/c mouse immunized three times with 100 µg GST-p57/c in CFA.; the animal had a strong antibody reponse at the time of sacrification. The supernatants of hybridomas were tested for BDV-specific antibodies by the indirect immunofluorescence assay (IFA) on persistently infected MDCK cells. Additionally, ELISA and Western blot analysis was performed. Hybridoma cells were cloned twice by picking single cells under a light microscope.

Polyvalent monospecific antiserum against the GST-p57/c fusion protein was obtained from a rabbit immunized subcutaneously with the GST-p57/c fusion protein as described above. This antisera was applied to persistently BDV-infected MDCK cells fixed in aceton (60 min at -20°C) or 4% paraformaldehyde (PFA) for 30 min at room temperature. The monospecific antiserum recognized virus-specific proteins in aceton-fixed cells scattered throughout the cytoplasm of infected MDCK cells. When the cells were fixed with PFA in order to stain for surface antigen, intensive staining was found

on the surface of BDV-infected MDCK cell. Furthermore, brain sections of experimentally BDV-infected rats were incubated with the monospecific and monoclonal anti-p57/c antisera. Viral antigen was detected mainly throughout the cytoplasm of infected neurons in the CNS of rats.

Example 4

ELISA

Screening of antibody-producing hybridomas and sera from BDV-infected rats was performed using recombinant GST-p57/c protein and GST as the control protein.

Ninety-six well microtiter plates (Greiner, Germany) were coated overnight at 4°C with 31 and 125 ng of recombinant GST-p57/c or GST protein per well in 50 µl of buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.20 g NaN_3 in 1000 ml H_2O). Plates were washed three times with washing buffer (0.5% Tween-20 in PBS) and incubated 1 hour with blocking buffer (0.5% gelatine, 1% BSA, 0.1% Thimerosal in PBS with 0.5% Tween-20) at room temperature. The microtiter plate was washed three times with washing buffer and 2 fold dilutions of the sera were prepared in the blocking buffer. 50 µl of the respective sera diluted from 1:20 to 1: 10240 was added to each well and incubated for 1 hour at room temperature. Plates were washed three times with washing buffer and biotin-conjugated rabbit anti-rat or anti-mouse IgG and IgM diluted 1:10 000 in blocking buffer were added to each well and incubated 1 hour at room temperature. After washing three times the plates were incubated with horseradish peroxidase conjugated to streptavidin (Amersham, Braunschweig), diluted 1:10 000 in blocking buffer for 1 hour at room temperature. After washing the plates three times, 200 µl of substrate solution was added to each well. The substrate solution consisted of 0.5 M Na_2PO_4, 0.1 M citric acid, 20 mg phenyldiamine and 20 ml 30% H_2O_2 in 50 ml H_2O. The plates were incubated for 5-10 min at room temperature and the reaction stopped by the addition of 50 µl sulphuric acid to each well. The absorbance at 492 nm was determined for each well using a microplate reader. Negative control wells without the primary antisera were used for calibration. The ELISA titer for each serum was defined as the endpoint dilution that yielded an optical density of 0.2. The results of this test using a convalescent and control rat serum are shown in Figure 6.

In order to establish a specific and sensitive ELISA for the recombinant BDV p57/c protein, the optimal antigen concentration was determined by checkerboard titration of positive and negative rat sera versus the following antigen concentrations: 31, 62, 125, 250 ng/well. The optimal concentration with the most linear response was 31 ng/well. The sensitivity of the ELISA system for the recombinant p57/c BDV-protein was established using sera from experimentally infected rats on days 40, 50 and 60 post infection (p.i.) known to be reactive by IFA (Titers ranging from 1:2280 to 1:5120) and Western blot analysis. All sera that has been found positive by these methods were also positive in the ELISA-system using the recombinant p57/c protein. The specificity was tested using sera from 5 noninfected rats and recombinant GST protein. Each ELISA proved to be highly specific for the detection of antibodies to the recombinant p57/c BDV-protein: at a dilution of 1:80 the noninfected rat sera had an OD-range from 0.026 to 0.051, the BDV-infected rat sera from 0.363 to 0.566. No nonspecific background was observed at dilutions 1:40 or higher.

Example 5

Cloning and expression of the p9.5 BDV-gene

The open reading frame of the p9.5 BDV-protein was amplified from cDNA of the B8 clone [VandeWoude et al., (1990) Science 250, p. 1278-1281] and from a field isolate of BDV (horse) using the following primers:

3' Primer (anti-sense) *GCGGAATTC* **TCATCATTC-GATAGCTGCTCCC** (Seq.-ID 8)
5' Primer (sense): *ATAGGATCC* **ATGAGTTC-CGACCTCCGGC** (Seq.-ID 9)

The conditions for the PCR reaction were described in example 1.

The amplified product was purified from agarose gels and cloned into the plasmid vector pGEX-2T (Pharmacia, Freiburg, Germany; Nr. 27-4801-01) after the restriction sites were cleaved using the restriction enzymes BamHI and EcoRI (Promega, Madison, USA). The viral gene was fused to the Glutathion-S-transferase (GST) gene of *Schistosoma japonicum* controlled by the *tac* promotor. The expression plasmid was transformed into competent *E. coli* Sure™-cells. Recombinant plasmids were analyzed using restriction analysis and DNA-sequencing methods. The DNA sequence of the cloned fragment (pGEX-p9.5) from the field isolate is shown in Figure 5.

Example 6

Expression and purification of the p9.5 BDV-protein in E. coli:

100 ml of pGEX-p9.5 containing *E. coli* were grown overnight in LB-medium with 0.1 mg/ml ampicillin (Serva, Heidelberg). This overnight culture was diluted in 1 liter of LB-medium with ampicillin and grown to log phase for 2-4 hours. The expression of the GST-p9.5 fusion protein was induced with IPTG (0.1 mM; Promega, Heidelberg, Germany) for 4 hours. The bacteria were pelleted by centrifugation (5900 g, 10 min, 4°C) and resuspended in PBS. The cells were lysed by sonication on ice and the cell debris pelleted by centrifugation (9800 g, 10 min, 4°C). The sonicated fusion

protein supernatant was added to an affinity matrix with glutathione (Glutathione Sepharose 4 B; Pharmacia, Nr. 27-4570-01). The purification of the GST-p9.5 fusion protein using Glutathione Sepharose 4B was done according to the manufacturer's protocol. The eluted fusion protein was dialyzed against 1xPBS for 24 hours at 4°C. The expression product was analyzed in SDS-PAGE and Immunoblot assays.

The expression of the virus-specific GST-p9.5 fusion protein by a recombinant pGEX-p.9.5 clone was analyzed in immunoblotting using *E.coli* lysates treated with and without IPTG. As a control an *E.coli* lysate transformed with the nonrecombinant pGEX-2T plasmid was used. The quality of the eluted fusion protein was analyzed in Western blot analyses using BDV-specific rat and rabbit antisera. The purified GST-p9.5 was easily detected by virus-specific antisera from rat and rabbit as a distinct band with a MW of ca. 35 kilodalton; 26 kd of the fusion protein represent the GST protein and ca. 9 kb represent the p9.5 BDV-protein.

Example 7

SDS-PAGE, SDS-PAGE-Tricin and Western blot analysis

10 ml of the purified recombinant GST-p9.5 and GST proteins, uninfected and BDV-infected OligoTL cell lysates as well as uninfected and BDV-infected rat brain homogenates were suspended in Laemmli sample buffer (Laemmli, 1970), heated for 2 min at 100°C, and separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 12 % polyacrylamide. The separated proteins were transferred to nitrocellulose membrane by electroblotting. Polyclonal antisera from rabbits and rats and monospecific rabbit anti-GST-p9.5 antisera were diluted 1:100 in PBS containing 0.5 % Tween-80 and 5% BSA. Nitrocellulose strips were incubated overnight at 4°C with the respective diluted antisera. After the strips were washed three times with PBS/0.5% Tween-20 (washing buffer) they were incubated with anti-species antibodies marked with biotin (Amersham, Braunschweig, Germany) in a dilution of 1:1000. After three washes with washing buffer the nitrocellulose strips were incubated with streptavidin conjugated horseradish peroxidase (Amersham, Braunschweig, Germany) diluted 1:2000 in washing buffer. Finally the strips were washed three times in PBS and stained in a solution of 0.5 mg/ml 4-chloro-1-naphtol, 20% (v/v) methanol and 0.4 ml/ml H_2O_2.

Tricin-SDS-PAGE gels were used for the separation of the affinity purified proteins; tricin allows the resolution of small proteins. Shortly, 12% acrylamid gels were prepared as described above. The anode buffer consisted of 0.2 M Tris (pH 8.9), the kathode buffer of 0.1 M Tris (pH8.25), 0.1 M Tricin and 0.1 % SDS (Schägger & Jagow, 1987). The separated proteins of the Tricin-SDS-PAGE gel were further analyzed by immunoblot technique as described above.

Example 8

Preparation of antisera

Polyvalent monospecific antiserum against the GST-p9.5 fusion protein was obtained from a rabbit immunized subcutaneously with 1 mg GST-p9.5 fusion protein in complete Freund's adjuvant (CFA). 4 and 8 weeks later the rabbit received booster immunizations with the same amount of antigen and was bled 1 week after the last immunization procedure. The sera was tested for their reactivity in indirect immunofluorescence assays on BDV-infected and uninfected MDCK cells as well as in Western blot analyses.

This antisera was applied to persistently BDV-infected MDCK cells fixed in aceton for 60 min at -20°C. The monospecific antiserum recognized virus-specific proteins mainly located in the nuclei of infected cells. This staining pattern was analogous to the reaction with monoclonal or monospecific antibodies specific for the p38 BDV-protein. Double immunofluorescence techniques using FITC and TRITC-labelled secondary antibodies revealed that the p9.5 BDV-protein colocalizes in the nucleus of infected cells with the p38 BDV-protein, the putative nucleoprotein of BDV. Furthermore, brain sections of experimentally BDV-infected rats were incubated with the monospecific anti-GST-p9.5 rabbit antiserum. Viral antigen was detected in the nucleus and cytoplasm of infected neurons in the CNS of rats.

Example 9

Antibody-mediated affinity chromatography

The procedure has been described by Haas et al. [J. Gen. Virol. 67 (1986), p. 235-241]. Shortly, sepharose CL-6B was treated with phoroglucinol and epichlorhydrin, activated with cyanogen bromide dissolved in acetonitrile, and conjugated with the gamma globulin fraction of the monospecific rabbit anti-GST-p9.5 serum at 4°C overnight. About 300 mg of protein was used per 10 ml of packed, activated sepharose. The column with the antibody-coated sepharose was equilibrated with PBS. After the application of the tissue or cell extracts, the column was washed extensively with PBS/1M NaCl and finally with Tris/NaCl (TN) buffer only. The material retained on the immunosorbent was eluted with PBS/1M $NaClO_4$. The eluate was concentrated by centrifugation dialysis using the Ultrafree-MC 10 kD-filters (Millipore, Germany) at 4°C.

In order to purify the p9.5 BDV-protein from BDV-infected cells, BDV-infected OligoTL cells were washed with PBS and scraped from the bottom of culture dish. The cell suspension was then washed and resuspended with PBS and sonicated three times for 10 seconds. The cell homogenate was centrifuged (5000 g, 10 min, 4°C) and the supernatant applied to the affinity col-

umn with anti-p9.5 antibodies. The column was washed and eluted as described above. Similarly, a 10% homogenate of a BDV-infected rat brain in TN-buffer was stirred for 1 hour at room temperature after the addition of 1% Triton X-100 and 0.5 % deoxycholate. The homogenate was centrifuged for 2 hours at 30 000 r.p.m. in a Beckman 45 Ti rotor to remove cell debris. The supernatant was applied to the affinity column and processed as described above.

The antibody-mediated affinity purification procedure with both antigen sources resulted clearly in the isolation of a virus-specific protein with a MW of approximately 9.5 kd; the 9.5 BDV-protein does not contain carbohydrate side chains as analyzed using a DIG glycon detection kit.

Example 10

ELISA

Screening of antibody-producing hybridomas and sera from BDV-infected rats was performed using recombinant GST-p9.5 protein and GST as the control protein.

Ninety-six well microtiter plates (Greiner, Germany) were coated overnight at 4°C with 31 and 125 ng of recombinant GST-p9.5 or GST protein per well in 50 µl of buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.20 g NaN_3 in 1000 ml H_2O). Plates were washed three times with washing buffer (0.5% Tween-20 in PBS) and incubated 1 hour with blocking buffer (0.5% gelatine, 1% BSA, 0.1% Thimerosal in PBS with 0.5% Tween-20) at room temperature. The microtiter plate was washed three times with washing buffer and 2 fold dilutions of the sera were prepared in the blocking buffer. 50 µl of the respective sera diluted from 1:20 to 1: 10240 was added to each well and incubated for 1 hour at room temperature. Plates were washed three times with washing buffer and biotin-conjugated rabbit anti-rat or anti-mouse IgG and IgM diluted 1:10 000 in blocking buffer were added to each well and incubated 1 hour at room temperature. After washing three times the plates were incubated with horseradish peroxidase conjugated to streptavidin (Amersham, Braunschweig), diluted 1:10 000 in blocking buffer for 1 hour at room temperature. After washing the plates three times, 200 µl of substrate solution was added to each well. The substrate solution consisted of 0.5 M Na_2PO_4, 0.1 M citric acid, 20 mg phenyldiamine and 20 ml 30% H_2O_2 in 50 ml H_2O. The plates were incubated for 5-10 min at room temperature and the reaction stopped by the addition of 50 µl sulphuric acid to each well. The absorbance at 492 nm was determined for each well using a microplate reader. Negative control wells without the primary antisera were used for calibration. The ELISA titer for each serum was defined as the endpoint dilution that yielded an optical density of 0.2. The results of this test using a convalescent and control rat serum are shown in Figure 7.

In order to establish a specific and sensitive ELISA for the recombinant BDV p9.5 protein, the optimal antigen concentration was determined by checkerboard titration of positive and negative rat sera versus the following antigen concentrations: 31, 62, 125, 250 ng/well. The optimal concentration with the most linear response was 31 ng/well. The sensitivity of the ELISA system for the recombinant p9.5 BDV-protein was established using sera from experimentally infected rats on days 40, 50 and 60 post infection (p.i.) known to be reactive by IFA (Titers ranging from 1:2280 to 1:5120) and Western blot analysis. All sera that has been found positive by these methods were also positive in the ELISA-system using the recombinant p9.5 protein. The specificity was tested using sera from 5 noninfected rats and recombinant GST protein. Each ELISA proved to be highly specific for the detection of antibodies to the recombinant p57/c BDV-protein: at a dilution of 1:80 the noninfected rat sera had an OD-range from 0.026 to 0.051, the BDV-infected rat sera from 0.363 to 0.566. No nonspecific background was observed at dilutions 1:40 or higher.

Claims

1. Polypeptide corresponding to the amino acid sequence of the protein p57 or p9.5 encoded by the Borna disease virus having a sequence of at least 10 consecutive amino acids of the amino acid sequence given in Figure 1 or Figure 2, respectively.

2. Polypeptide according to claim 1 characterized in that the protein p57 or p9.5, respectively, is encoded by a field isolate of Borna disease virus.

3. Polypeptide according to claim 1 or 2 characterized in that the polypeptide has a sequence of at least 25 consecutive amino acids of the amino acid sequence given in Figure 1 or Figure 2, respectively.

4. Polypeptide according to any of claims 1 to 3 characterized in that the polypeptide has a sequence of at least 50 consecutive amino acids of the amino acid sequence given in Figure 1 or Figure 2, respectively.

5. Polypeptide according to any of claims 1 to 4 characterized in that the polypeptide has a sequence of not more than 80 consecutive amino acids of the amino acid sequence given in Figure 1 or Figure 2, respectively.

6. Testkit for the determination of antibodies directed against Borna disease virus in a sample comprising at least one polypeptide according to claims 1 to 5 and a label for the detection of the complex formed by the polypeptide and the antibodies to be determined.

7. Testkit according to claim 6 characterized in that the label is linked to an antibody which specifically binds to the antibody to be determined.

8. Testkit according to claim 6 characterized in that it is a kit for performing an ELISA, Western blot, RIA or dot blot test.

9. Testkit according to claim 7 characterized in that the label is an enzyme which can catalyze a reaction resulting in a coloured end product.

10. Method for determining an infection by Borna disease virus comprising

a) contacting a sample to be determined with at least one polypeptide according to claims 1 to 5 whereby the polypeptide binds to antibodies elicited by a former infection of Borna disease virus and

b) determining the binding of said polypeptide to the specific antibodies which are present in the sample to be tested.

11. Isolated DNA fragment characterized in that said DNA encodes a polypeptide according to claim 1 to 5 whereby the DNA fragment comprises not more than 240 base pairs.

12. Isolated DNA fragment according to claim 11 characterized in that the DNA fragment comprises not more than 150 base pairs.

13. Isolated DNA fragment according to claims 11 or 12 characterized in that the DNA sequence corresponds to a part of the sequence of Figure 4 or 5, respectively.

14. Isolated RNA fragment characterized in that said RNA fragment encodes a polypeptide according to claims 1 to 5 with the proviso that the RNA fragment comprises not more than 240 base pairs.

15. Use of a polypeptide according to claims 1 to 5 for the production of a vaccine.

16. Use of a DNA fragment according to claims 11 to 13 for nucleic acid immunization.

17. Use of an RNA fragment according to claim 14 for nucleic acid immunization.

Figure 1

```
    Met Gln Pro Ser Met Ser Phe Leu Ile Gly Phe Gly Thr Leu
Val Leu
      1               5                  10
15

    Ala Leu Ser Ala Arg Thr Phe Asp Leu Gln Gly Leu Ser Cys
Asn Thr
                  20                  25                  30

    Asp Ser Thr Pro Gly Leu Ile Asp Leu Glu Ile Arg Arg Leu
Cys His
             35                  40                  45

    Thr Pro Thr Glu Asn Val Ile Ser Cys Glu Val Ser Tyr Leu
Asn His
          50                  55                  60

    Thr Thr Ile Ser Leu Pro Ala Val His Thr Ser Cys Leu Lys
Tyr His
      65                  70                  75
80

    Cys Lys Thr Tyr Trp Gly Phe Phe Gly Ser Tyr Ser Ala Asp
Arg Ile
                  85                  90
95

    Ile Asn Arg Tyr Thr Gly Thr Val Lys Gly Cys Leu Asn Asn
Ser Ala
                 100                 105                 110

    Pro Glu Asp Pro Phe Glu Cys Asn Trp Phe Tyr Cys Cys Ser
Ala Ile
             115                 120                 125

    Thr Thr Glu Ile Cys Arg Cys Ser Ile Thr Asn Val Thr Val
Ala Val
         130                 135                 140

    Gln Thr Phe Pro Pro Phe Met Tyr Cys Ser Phe Ala Asp Cys
Ser Thr
    145                 150                 155
160

    Val Ser Gln Gln Glu Leu Glu Ser Gly Lys Ala Met Leu Ser
Asp Gly
                 165                 170
175
```

Ser Thr Leu Thr Tyr Thr Pro Tyr Ile Leu Gln Ser Glu Val Val Asn
180 185 190

Arg Thr Leu Asn Gly Thr Ile Leu Cys Asn Ser Ser Ser Lys Ile Val
195 200 205

Ser Phe Asp Glu Phe Arg Arg Ser Tyr Ser Leu Thr Asn Gly Ser Tyr
210 215 220

Gln Ser Ser Ser Ile Asn Val Thr Cys Ala Asn Tyr Thr Ser Ser Cys
225 230 235 240

Arg Pro Arg Leu Lys Arg Arg Arg Arg Asp Thr Gln Gln Ile Glu Tyr
245 250 255

Leu Val His Lys Leu Arg Pro Thr Leu Lys Asp Ala Trp Glu Asp Cys
260 265 270

Glu Ile Leu Gln Ser Leu Leu Leu Gly Val Phe Gly Thr Gly Ile Ala
275 280 285

Ser Ala Ser Gln Phe Leu Arg Gly Trp Leu Asn His Pro Asp Ile Val
290 295 300

Gly Tyr Ile Val Asn Gly Ile Gly Val Val Trp Gln Cys His Arg Val
305 310 315 320

Asn Val Thr Phe Met Ala Trp Asn Glu Ser Thr Tyr Tyr Pro Pro Val
325 330 335

Asp Tyr Asn Gly Arg Lys Tyr Phe Leu Asn Asp Glu Gly Arg Leu Gln
340 345 350

Thr Asn Thr Pro Glu Ala Arg Pro Gly Leu Lys Arg Val Met Trp Phe
355 360 365

Gly Arg Tyr Phe Leu Gly Thr Val Gly Ser Gly Val Lys Pro Arg Arg
370 375 380

Ile Arg Tyr Asn Lys Thr Ser Arg Asp Tyr His Leu Glu Glu Phe Glu
385 390 395 400

Ala Ser Leu Asn Met Thr Pro Gln Thr Ser Ile Ala Ser Gly His Glu
405 410 415

Thr Asp Pro Ile Asn His Ala Tyr Gly Thr Gln Ala Asp Leu Leu Pro
420 425 430

Tyr Thr Arg Ser Ser Asn Ile Thr Ser Thr Asp Thr Gly Ser Gly Trp
435 440 445

Val His Ile Gly Leu Pro Ser Phe Ala Phe Leu Asn Pro Leu Gly Trp
450 455 460

Leu Arg Asp Leu Leu Ala Trp Ala Ala Trp Leu Gly Gly Val Leu Tyr
465 470 475 480

Leu Ile Ser Leu Cys Val Ser Leu Pro Ala Ser Phe Ala Arg Arg Arg
485 490 495

Arg Leu Ala Arg Trp Gln Glu
500

Figure 2

```
    Met Ser Ser Asp Leu Arg Leu Thr Leu Leu Glu Leu Val Arg
Arg Leu
      1               5                  10
15

    Asn Gly Asn Ala Thr Ile Glu Ser Gly Arg Leu Pro Gly Gly
Arg Arg
                20                  25                  30

    Arg Ser Pro Asp Thr Thr Thr Gly Thr Ile Gly Val Ala Lys
Thr Thr
            35                  40                  45

    Glu Asp Pro Lys Glu Cys Ile Asp Pro Thr Ser Arg Pro Ala
Pro Glu
        50                  55                  60

    Gly Pro Gln Glu Glu Pro Leu His Asp Leu Arg Pro Arg Pro
Ala Asn
     65                  70                  75
80

    Arg Lys Gly Ala Ala Val Glu
                    85
```

Figure 3

```
    Met Tyr Cys Ser Phe Ala Asp Cys Ser Thr Val Ser Gln Gln
Glu Leu
      1               5                  10
15

    Glu Ser Gly Lys Ala Met Leu Ser Asp Gly Ser Thr Leu Thr
Tyr Thr
                20                  25                  30

    Pro Tyr Ile Leu Gln Ser Glu Val Val Asn Arg Thr Leu Asn
Gly Thr
           35                  40                  45

    Ile Leu Cys Asn Ser Ser Ser Lys Ile Val Ser Phe Asp Glu
Phe Arg
         50                  55                  60

    Arg Ser Tyr Ser Leu Thr Asn Gly Ser Tyr Gln Ser Ser Ser
Ile Asn
      65                  70                  75
80

    Val Thr Cys Ala Asn Tyr Thr Ser Ser Cys Arg Pro Arg Leu
Lys Arg
                85                  90
95

    Arg Arg Arg Asp Thr Gln Gln Ile Glu Tyr Leu Val His Lys
Leu Arg
             100                 105                 110

    Pro Thr Leu Lys Asp Ala Trp Glu Asp Cys Glu Ile Leu Gln
Ser Leu
          115                 120                 125

    Leu Leu Gly Val Phe Gly Thr Gly Ile Ala Ser Ala Ser Gln
Phe Leu
       130                 135                 140

    Arg Gly Trp Leu Asn His Pro Asp Ile Val Gly Tyr Ile Val
Asn Gly
    145                 150                 155
160

    Ile Gly Val Val Trp Gln Cys His Arg Val Asn Val Thr Phe
Met Ala
             165                 170
175

    Trp Asn Glu Ser Thr Tyr Tyr Pro Pro Val Asp Tyr Asn Gly
Arg Lys
          180                 185                 190
```

Tyr Phe Leu Asn Asp Glu Gly Arg Leu Gln Thr Asn Thr Pro Glu Ala
195 200 205

Arg Pro Gly Leu Lys Arg Val Met Trp Phe Gly Arg Tyr Phe Leu Gly
210 215 220

Thr Val Gly Ser Gly Val Lys Pro Arg Arg Ile Arg Tyr Asn Lys Thr
225 230 235 240

Ser Arg Asp Tyr His Leu Glu Glu Phe Glu Ala Ser Leu Asn Met Thr
245 250 255

Pro Gln Thr Ser Ile Ala Ser Gly His Glu Thr Asp Pro Ile Asn His
260 265 270

Ala Tyr Gly Thr Gln Ala Asp Leu Leu Pro Tyr Thr Arg Ser Ser Asn
275 280 285

Ile Thr Ser Thr Asp Thr Gly Ser Gly Trp Val His Ile Gly Leu Pro
290 295 300

Ser Phe Ala Phe Leu Asn Pro Leu Gly Trp Leu Arg Asp Leu Leu Ala
305 310 315 320

Trp Ala Ala Trp Leu Gly Gly Val Leu Tyr Leu Ile Ser Leu Cys Val
325 330 335

Ser Leu Pro Ala Ser Phe Ala Arg Arg Arg Arg Leu Ala Arg Trp Gln
340 345 350

Glu

Figure 4

ATGCAGCCTT CAATGTCTTT TCTTATCGGC TTCGGAACAT TGGTGTTGGC CCTCTCGGCC 60

CGGACATTCG ATCTTCAGGG CCTTAGTTGC AATACTGACT CCACTCCTGG ACTGATCGAC 120

CTGGAGATAA GGCGACTTTG CCACACCCCA ACGGAAAATG TCATTTCATG CGAGGTTAGT 180

TATCTTAACC ACACGACTAT TAGCCTCCCG GCAGTCCACA CATCATGCCT CAAGTACCAC 240

TGCAAAACCT ATTGGGGATT CTTTGGTAGT TACAGCGCTG ACCGAATCAT CAATCGGTAC 300

ACTGGTACTG TTAAGGGTTG TTTAAACAAC TCAGCACCAG AAGACCCCTT CGAGTGCAAC 360

TGGTTCTACT GCTGCTCGGC GATTACAACA GAAATCTGCC GATGCTCTAT TACAAATGTC 420

ACAGTGGCTG TACAAACATT CCCACCGTTT ATGTACTGCA GCTTTGCGGA CTGTAGCACC 480

GTGAGTCAGC AGGAGCTAGA GAGTGGAAAG GCAATGCTGA GCGATGGCAG CACATTAACT 540

TATACCCCTT ATATCTTACA GTCAGAAGTC GTGAACAGAA CCCTTAATGG GACCATACTC 600

TGCAACTCAT CCTCCAAGAT AGTTTCCTTT GATGAATTTA GGCGTTCATA CTCCCTAACG 660

AATGGTAGTT ACCAGAGCTC ATCAATCAAT GTGACGTGTG CAAACTACAC GTCGTCCTGC 720

CGGCCCAGGT TGAAAAGGCG GCGTAGGGAC ACCCAGCAGA TTGAGTATCT AGTTCACAAG 780

CTTAGGCCCA CACTGAAAGA TGCATGGGAG GACTGTGAGA TCCTCCAGTC TCTGCTCCTA 840

GGGGTGTTTG GTACTGGGAT CGCAAGTGCT TCTCAATTTT TGAGGGGCTG GCTCAACCAC 900

CCTGACATCG TCGGGTATAT AGTTAATGGA ATTGGGGTTG TCTGGCAATG CCATCGTGTT 960

AATGTCACAT TCATGGCGTG GAATGAGTCC ACATATTACC CTCCAGTAGA TTACAATGGG 1020

CGGAAGTACT TTCTGAATGA TGAGGGGAGG CTACAAACAA ACACCCCCGA GGCGAGGCCA 1080

GGGCTAAAGC GGGTCATGTG GTTCGGTAGG TACTTCCTAG GGACAGTAGG GTCTGGGGTG 1140

AAACCGAGGA GGATTCGGTA CAATAAGACT TCACGTGACT ACCACCTAGA GGAGTTTGAG 1200

GCAAGTCTCA ACATGACCCC CCAGACCAGT ATCGCTTCAG GTCATGAGAC AGACCCCATA 1260

AATCATGCCT ACGGAACGCA GGCTGATCTC CTTCCATACA CCAGGTCTAG TAATATAACG 1320

TCTACAGATA CAGGCTCAGG CTGGGTGCAC ATCGGCCTAC CCTCATTTGC CTTCCTCAAT 1380

CCCCTCGGGT GGCTCAGGGA CTTACTTGCA TGGGCGGCCT GGTTGGGTGG GGTCCTATAC 1440

TTAATAAGTC TTTGTGTTTC CTTACCAGCC TCCTTCGCGA GGAGGAGACG CCTCGCGCGG 1500

TGGCAGGAAT AA 1512

Figure 5

```
    ATGAGTTCCG ACCTCCGGCT GACATTGCTT GAACTAGTCA GGAGGCTCAA
TGGCAACGCG        60

    ACCATCGAGT CTGGTCGACT CCCTGGAGGA CGAAGAAGAT CCCCAGACAC
TACGACGGGA        120

    ACGATCGGGG TCACCAAGGC CACGGAAGAT CCCAAGGAAT GCATTGACCC
AACCAGTCGA        180

    CCAGCTCCTG AAGGACCTCA GGAAGAACCC CTCCATGATC TCAGACCCAG
ACCAGCGAAC        240

    CGGAAGGGAG CAGCTGTCGA ATGA
264
```

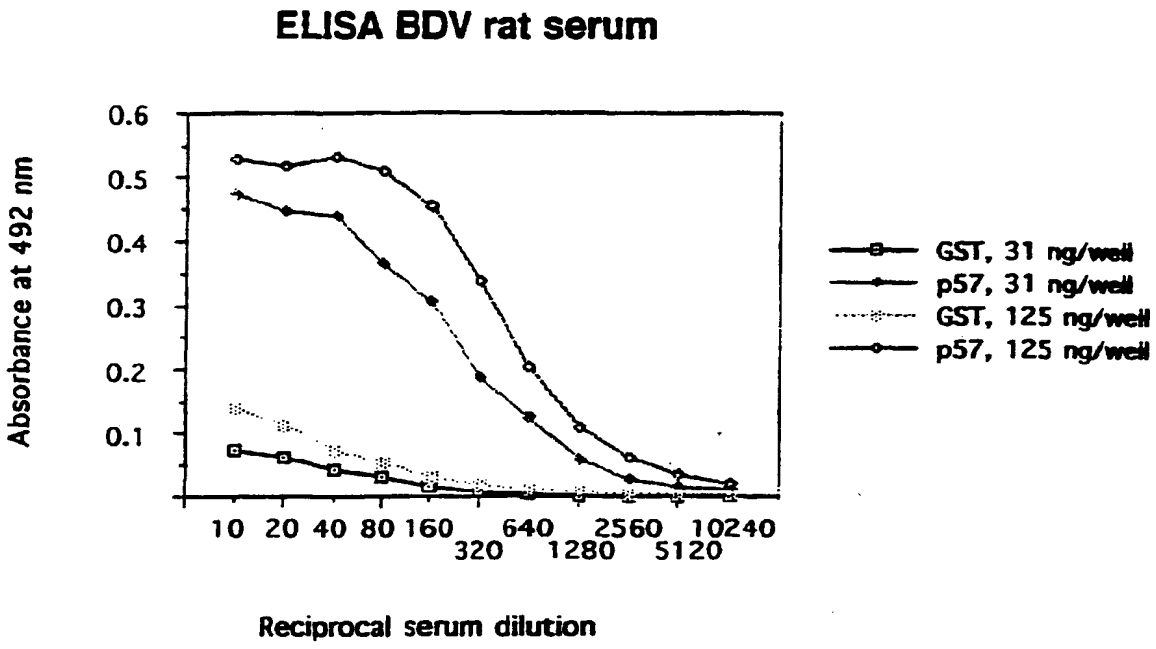

Figure 6

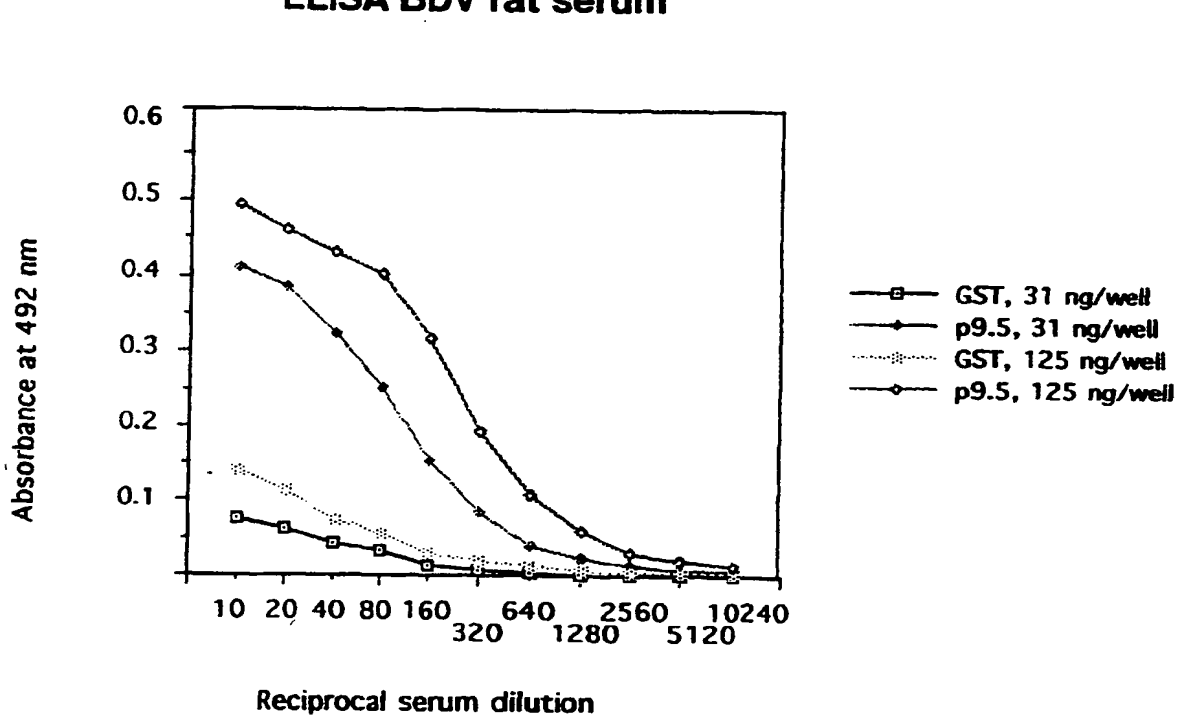

Figure 7

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EUROPEAN SEARCH REPORT

Application Number
EP 96 10 2575

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,X	JOURNAL OF VIROLOGY, vol. 68, no. 3, March 1994, pages 1382-1396, XP002003339 BEATRICE CUBITT ET AL.: "Sequence and genome organization of Borna disease virus"	1-5	C12N15/40 C07K14/00 G01N33/569 A61K39/12 A61K48/00
Y		6-10	
A	* abstract * * page 1382, right-hand column, paragraph 1 * * page 1384, left-hand column, last paragraph - page 1390, right-hand column, paragraph 1; figure 2 * * page 1391, left-hand column, paragraph 3 - right-hand column, paragraph 1 * * page 1393, left-hand column, paragraph 2 - right-hand column, paragraph 2 * ---	11-14	
D,Y	JOURNAL OF CLINICAL MICROBIOLOGY, vol. 33, no. 2, February 1995, pages 348-351, XP000571175 THOMAS BRIESE ET AL.: "Enzyme-linked immunosorbent assay for detecting antibodoes to Borna disease virus-specific proteins" * abstract * * page 348, left-hand column, paragraph 2 * * page 349, left-hand column, paragraph 3 - page 350, right-hand column, paragraph 3 * --- -/--	6-10	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)
C07K
C12N
G01N
A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	21 August 1996	Montero Lopez, B

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& : member of the same patent family, corresponding document

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Application Number
EP 96 10 2575

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,A	SCIENCE (WASHINGTON, D. C., 1883-) (1990), 250(4985), 1278-81 CODEN: SCIEAS;ISSN: 0036-8075, 1990, XP002003342 VANDEWOUDE, SUSAN ET AL: "A Borna virus cDNA encoding a protein recognized by antibodies in humans with behavioral diseases" * abstract * * page 1279, left-hand column, paragraph 2 - right-hand column, paragraph 1; figure 3 * * page 1279, right-hand column, paragraph 3 - page 1281, middle column, paragraph 1 * ---	1-7, 11-14	
A	VIROLOGY (1993), 195(1), 229-38 CODEN: VIRLAX;ISSN: 0042-6822, 1993, XP002003350 PYPER, J. M. ET AL: "Genomic organization of the structural proteins of borna disease virus revealed by a cDNA clone encoding the 38-kDa protein" * abstract * * page 229, right-hand column, paragraph 2 - page 230, left-hand column, paragraph 2 * * page 232, right-hand column, paragraph 4 - page 233, right-hand column, paragraph 1; figures 3,4 * --- -/--	1-5, 11-14	TECHNICAL FIELDS SEARCHED (Int.Cl.6)

The present search report has been drawn up for all claims

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Application Number
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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	JOURNAL OF VIROLOGY, vol. 68, no. 8, August 1994, pages 5007-5012, XP002003341 PATRICK A. SCHNEIDER ET AL.: "RNA splicing in Borna disease virus, a nonsegmented, negative-strand RNA virus " * page 5007, left-hand column, paragraph 1 * * page 5011, left-hand column, paragraph 4 - right-hand column, paragraph 2 * ---	11-14	
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TECHNICAL FIELDS SEARCHED (Int.Cl.6)

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